Dissociation between Transmissible Spongiform Encephalopathy (TSE) Infectivity and Proteinase K-Resistant PrPSc Levels in Peripheral Tissue from a Murine Transgenic Model of TSE Disease

Dissociation between TSE infectivity and PrP-res levels in peripheral tissue from a murine transgenic model of TSE disease.

Running title – High peripheral infectivity with low PrP-res

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ABSTRACT

Most current diagnostic tests for transmissible spongiform encephalopathies (TSE) rely on the presence of PK-resistant PrP^{Sc} (PrP-res) in post-mortem tissues as indicative of TSE disease. However, a number of studies have highlighted a discrepancy between TSE infectivity and PrP-res levels in both natural and experimental cases of TSE disease. Previously we have shown high TSE infectivity levels in the brain tissue of mice that have a clinical TSE disease with associated vacuolar pathology but little or no PrP-res detectable. Here the levels of TSE infectivity and PrP-res within a peripheral tissue of this mouse model were investigated. Biochemical analysis identified low levels of PrP-res were present in the spleen tissue in comparison to levels observed in the spleen of mice infected with ME7 or 79A. However, upon sub-passage of brain and spleen tissue from clinically ill mice with little or no PrP-res detectable, similar short incubation periods to disease were observed, indicating that infectivity levels were similarly high in both tissues. Thus the discrepancy between PrP-res and TSE infectivity was also present in the peripheral tissues of this disease model. This result indicates that peripheral tissues can contain higher levels of infectivity given the correct combination of host species, PrP genotype and TSE agent. Therefore the assumption that levels of peripheral infectivity are lower than those in the central nervous system is not always correct and could have implications for current food safety regulations.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that can affect both humans and animals. TSEs can be sporadic, familial or acquired diseases. The nature of the infectious agent remains unknown; however the prion hypothesis proposes that a misfolded form (PrP^{Sc}) of the host glycoprotein, PrP^{C}, acts as the sole or main component of the infectious agent in TSE disease (1). The abnormal, disease associated form is increased in β-sheet content, detergent insoluble and partially resistant to proteinase K (PK) digestion. Based on the prion hypothesis the majority of current diagnostic tests rely on the protease-resistance of the disease-associated form (PrP-res) to identify cases of TSE disease post-mortem. However, a number of experimental cases of TSE disease have been identified in which PrP-res was not detectable, including wild-type mice infected with BSE (2) and wild-type mice infected with hamster scrapie (3). In both cases, sub-passage of the tissue confirmed the presence of TSE infectivity despite the absence of PrP-res.

The highest levels of TSE infectivity are found in tissues of the central nervous system (CNS) of infected animals. However, the infectious agent has also been demonstrated to be present in peripheral tissues of some animals, specifically in the lymphoreticular system (LRS) including the spleen, with the presence of PrP-res and/or TSE infectivity during disease pathogenesis dependent upon host species, PrP genotype and strain of TSE agent. In scrapie-infected sheep, VRQ/VRQ homozygotes have PrP-res deposition in the spleen whilst other genotypes, ARR/ARR and VRQ/ARR do not show any PrP-res accumulation in the spleen (4). In terminal BSE-infected cattle no PrP-res has been detected in the spleen tissue (5, 6), indicating that the LRS is not involved in the disease pathogenesis of BSE in cattle. However, in sheep experimentally infected with BSE, TSE infectivity and PrP^{Sc} has been identified in the peripheral

tissues including the LRS and the spleen (7, 8). In murine models, infectivity levels in the spleen tissue have been shown to have an initial increase in titre within days of inoculation that then plateaus for the remainder of the disease duration, long before infectivity is detected in the CNS (9, 10). At disease end-point, the spleen contains lower titres of infectivity than the corresponding brain tissue, regardless of the route of infection. For example, it has been shown that wild-type/139A mouse spleens contain 2-3$\log_{10}$ units less infectivity than the brain (11). In correlation with the infectivity data, the levels of PrP-res in the spleen of 139A infected mice were 500-fold less than those observed in the brain tissue at terminal stage of disease (11). Further studies identified that the level of PrP-res was 200-300 fold less in the spleen than in the brain of mice infected with a mouse-adapted strain of Gerstmann-Sträussler-Scheinker (GSS) originating from Fukuoka-1 GSS case (12).

If peripheral levels of infectivity are equally low in all cases of TSE disease, the current safety measures in place for removal of specified risk material should prevent any infected material from entering the food chain for human consumption. However, a number of natural and experimental TSE isolates have been identified that do not have the expected association between levels of TSE infectivity and PrP-res in all tissues (2, 3, 13-15). Indeed one study of atypical scrapie in sheep identified the presence of high levels of TSE infectivity in the spleen tissue, despite the absence of abnormal PrP (13). Given that the majority of current diagnostic tests rely on the presence of PrP-res as indicative of TSE disease, disease cases and tissues where PrP-res is absent may be falsely identified as negative. Further, the disparity between PrP-res and TSE infectivity levels may result in an underestimation of infectivity levels that are present in peripheral tissues that are allowed to enter the food chain, increasing the potential for zoonotic transfer.

We have previously identified a unique mouse model of infectious TSE disease that highlights the discrepancy between PrP-res levels and corresponding TSE infectious titres (14, 16, 17). Previous work has performed extensive characterisation of the relationship between PrP-res and TSE infectivity in the brain tissue of these disease models. Here we investigate the relationship between PrP-res and TSE infectivity within the peripheral spleen tissue of these mice using both biochemical analyses and mouse bioassay, and demonstrate that extremely high levels of infectivity can be present in peripheral tissue without a corresponding increase in PrP-res. If peripheral tissues can be as infectious as brain tissue this could represent an increased risk to food safety. Indeed if the correct combination of host species, PrP genotype and strain of agent was to occur naturally that resulted in high peripheral infectivity, infected peripheral tissue could have the potential to enter the food chain.

MATERIALS AND METHODS

Ethics Statement

All experimental protocols were submitted to the Local Ethical Review Committee for approval before mice were inoculated. All experiments were performed under license and in accordance with the UK Home Office Regulations (Animals (Scientific Procedures) Act of 1986).

Primary transmission to 101LL transgenic mice

Inbred gene-targeted transgenic mouse line 101LL and the wild-type 129/Ola control line have been previously described (16). 101LL/GSS tissues were produced by inoculation of 101LL mice with 1% (w/v) brain homogenate from the frontal cortex of a P102L GSS brain that had confirmed clinical GSS disease and abundant PrP-res detectable by immunoblot (Provided by Prof J Ironside, National CJD Surveillance and Research Unit (NCJDSRU), Edinburgh, UK). 101LL/263K tissues were produced by inoculation of 101LL transgenic mice with 1% (w/v) brain homogenate from a 263K-infected hamster. As P102L GSS and 263K do not transmit efficiently to wild type mice (16, 17) alternative controls were selected to provide tissue from models in which the relationship between PrP^{Sc} and infectivity in the periphery had been previously examined (ME7 and 79A mouse adapted scrapie).

Clinical assessment and vacuolation scoring

Mice were assessed for the presence of clinical disease as previously described (18) and were culled by cervical dislocation when either a clinical TSE disease or an inter-current illness was observed. All experiments were performed under license and in accordance with the UK Home Office Regulations (Animals (Scientific Procedures) Act of 1986). Brain and spleen tissue was

recovered at post-mortem for biochemical and immunohistochemical analysis. Half-brain sections (6μm) were stained using haematoxylin and eosin and the abundance of TSE-related vacuolation was assessed at nine grey-matter regions (medulla, cerebellum, superior colliculus, hypothalamus, thalamus, hippocampus, septum, retrospinal cortex, cingulated and motor cortex) and three regions of white matter (cerebellar white matter, midbrain white matter and cerebral peduncle) as described previously (10). DNA was extraction from tail tissue that was taken post mortem. PCR and restriction enzyme digestion was performed as previously described (16) to confirm the presence of the P101L mutation.

Sub-passage inoculations

Inocula were prepared from the brains and spleens of two 101LL/GSS mice (designated 101LL/GSS(a) and 101LL/GSS(b)) and two 101LL/263K mice (designated 101LL/263K(c) and 101LL/263K(d)) that all had confirmed clinical TSE disease with associated TSE-vacuolar pathology. Inoculum was prepared from a wild-type/79A mouse brain and spleen tissue as controls (designated wild-type/79A(e)). A 10% (w/v) homogenate of each tissue was prepared in sterile saline and was used to produce a dilution series of 10^{-2}, 10^{-3} and 10^{-4} for each homogenate. Utilising previous data (14) it was hypothesised that a 10^{-4} dilution of brain homogenate would produce a 100% attack rate. Given the expected 2-3$\log_{10}$ difference in TSE infectivity titres between brain and spleen tissues in murine models (11), the inoculation of these three dilutions was predicted to allow the identification of difference in incubation period between tissues, and provide an estimation in difference in titre. Each dilution (20μl) was inoculated intra-cerebrally under anaesthesia into groups of 101LL mice for 101LL/GSS and 101LL/263K inocula and wild-type 129/Ola mice for wild-type/79A inocula. Dilutions of brain homogenate were inoculated into groups of 6 mice at 10^{-2} and 10^{-3} dilutions and 8 mice at 10^{-4}

dilution. Dilutions of spleen homogenate were inoculated into groups of 6 mice at 10^{-2} dilution and 8 mice at 10^{-3} and 10^{-4} dilution due to the predicted lower titres.

Immunohistochemistry to detect PrP deposition

Immunohistochemistry was performed to detect PrP deposition in the brain tissue. Briefly, following fixation in 10% formal saline, brains were treated for 1.5 hours in 98% formic acid, dissected and embedded in paraffin. Sections (6µm) of brain tissue were hydrated, autoclaved for 15 minutes at 121˚C and incubated in 98% formic acid for 5 minutes to expose the PrP epitopes. Sections were incubated in 1% hydrogen peroxide/methanol and washed in 0.2% BSA/PBS. Sections were blocked with normal goat serum, incubated overnight in anti-PrP monoclonal antibody 6H4 (Prionics), (0.5µg/ml) and then with secondary anti-mouse biotinylated antibody (Jacksons Immuno Research Laboratories) (2µg/ml) for 1 hour. Sections were processed using the ABC Elite kit (Vector Laboratories) and the signal was visualised by a reaction with hydrogen perozidize-activated diaminobenzidine.

Precipitation of PrP-res with NaPTA from primary spleen tissue

Sodium phosphotungstic acid (NaPTA) precipitation has previously been shown to increase the sensitivity of immunoblotting for the detection of PrP^{Sc} (19). All incubations were performed at 37˚C with agitation. Briefly, spleen tissue from terminal animals was homogenised in 0.1M Tris-HCl (pH7.4) to a 10% (w/v) homogenate with a glass dounce homogeniser. Cellular debris was removed through centrifugation at 1000 rpm for 5 minutes. An equal volume of 2% Sarkosyl/0.1M Tris-HCl was added to the supernatant and incubated for 10 minutes. Homogenates were digested with 50µg/ml PK for 1 hour. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to 2mM. PrP-res was selectively precipitated with 0.3% (w/v)

NaPTA for 20 minutes. Homogenates were centrifuged 15,400g for 30 minutes. Pellets were washed with 83mM EDTA, 0.1% Sarkosyl/0.1M Tris-HCl and a further 30 minutes centrifugation (15,400g) produced the PrP-res pellet that was re-suspended in the appropriate volume of tris-glycine sample buffer (Invitrogen) according to the starting weight of the tissue.

Precipitation of PrP-res with NaPTA from residual inocula and brain tissue of recipient mice

All incubations were performed at 37˚C with agitation to allow precipitation of PrP-res from residual inocula. Briefly, to a 50µl aliquot of each 10% (w/v) homogenate, 0.1M magnesium chloride was added and incubated for 15 minutes. An equal volume of 4% Sarkosyl/0.1M Tris-HCl was added to the supernatant and incubated for 5 minutes. Homogenates were digested with 50µg/ml PK for 1 hour and precipitation of PrP-res with NaPTA performed as described for spleen tissues. The PrP-res pellet was re-suspended in 15µl tris-glycine sample buffer (Invitrogen) according to the starting weight of the tissue.

Immunoblotting of PrP-res

PrP-res pellets produced from NaPTA precipitation were diluted as described in the text. Samples were loaded on 12% tris/glycine polyacrylamide gels (Invitrogen) and separated by SDS-PAGE. Separated proteins were transferred onto a polyvinylidenedifluoride membrane by the semi-dry transfer system (BIO-RAD). PrP was detected with monoclonal antibody 8H4 (Sigma), using chemiluminescent solution (West Dura ECL substrate, Pierce) with images captured onto x-ray film.

RESULTS

Low levels of PrP-res in the spleen tissue of 101LL/GSS and 101LL/263K mice.

Spleens were harvested at cull from several 101LL transgenic mice that had been inoculated with P102L GSS or hamster 263K scrapie. All spleens selected for analysis were from mice which showed clinical signs of TSE disease and confirmed vacuolar pathology, but had low levels of PrP deposition by immunohistochemical analysis in the brain tissue. The levels of PrP-res in the spleens of infected mice were investigated to determine if PrP conversion and therefore replication of infectivity was occurring in the spleen tissue of these unique disease models. It has previously been shown that it is necessary to concentrate the spleen material in order to detect disease-associated forms of PrP (20) and to minimise IgG cross-reactivity during the immunoblot procedure. Therefore in these studies, PrP-res in spleen tissue was precipitated using sodium phosphotungstic acid (NaPTA) (21) to concentrate the PrP-res present for detection by immunoblotting. Analysis of the PK digested, NaPTA precipitated material identified a variation in the level of PrP-res between individual spleens (Figure 1A). However, the level of PrP-res in some 101LL/GSS and 101LL/263K spleens appeared to be lower than the level of PrP-res in the wild-type/ME7 spleens from which PrP-res was isolated as a control (Figure 1A). In order to confirm the difference in levels, doubling dilutions of the PK digested, NaPTA precipitated material from 101LL/GSS and 101LL/263K and a wild-type/ME7 spleen were analysed to determine the minimum tissue equivalent at which PrP-res could be detected with a semi-quantitative immunoblot (Figure 1B). 101LL/GSS spleens were shown to contain PrP-res at a level similar to the PrP-res levels in a wild-type/ME7 spleen albeit with a lighter band intensity. 101LL/263K spleens contained PrP-res present at a level two fold lower than that observed in the wild-type/ME7 spleens. Importantly of all the spleen tissue analysed, 3/20 101LL/GSS mice and

194 1/12 101LL/263K mice analysed contained no detectable PrP-res even with NaPTA
195 precipitation.

196 *Brain and spleen tissues show equivalent high levels of infectivity in 101LL/GSS and*
197 *101LL/263K mice.*

198 In order to confirm the presence of infectivity in spleen tissue and compare the levels of
199 infectivity present in brain and spleen from the same mouse, 10% (w/v) homogenates of a brain
200 and spleen from two 101LL/GSS and two 101LL/263K mice were prepared and used to produce
201 a dilution series (10^{-2}, 10^{-3} and 10^{-4}) for inoculation. 101LL/GSS and 101LL/263K brain and
202 spleen homogenates were inoculated into groups of 101LL transgenic mice. As both P102L GSS
203 and hamster 263K scrapie had been shown previously to be poorly transmissible to wild type
204 mice (16, 17), brain and spleen homogenates from murine 79A scrapie were inoculated as
205 controls. Average incubation periods to disease were similar for each brain and spleen inocula
206 pair from 101LL/GSS and 101LL/263K mice (Figure 2). No statistical differences (Student's t-
207 test) were observed between the average incubation periods to disease at each dilution for
208 101LL/263K. Although no statistical differences between incubation periods were observed for
209 the majority of dilutions of 101LL/GSS, the two exceptions were 101LL/GSS(a) 10^{-3} dilution of
210 brain homogenate that had a significantly (p-value 8.3×10^{-5}) shorter incubation period to disease
211 than the spleen homogenate at the same dilution, and 101LL/GSS(b) 10^{-2} dilution that had a
212 significantly (p-value 5.6×10^{-4}) shorter incubation period to disease from the brain homogenate
213 than the spleen homogenate. However, these significant differences were not consistent and were
214 not observed for all dilutions of the same inoculum. Overall the data (Figure 2) show that the
215 brain and spleen incubation periods were similar in 101LL/GSS and 101LL/263K tissues. In
216 comparison, significantly different incubation periods to disease were observed between brain

and spleen homogenates from wild-type/79A mice at each dilution. The average incubation period to disease for the wild-type/79A spleen homogenates was 30-40 days longer than the average incubation period to disease with the corresponding brain homogenates.

Analysis of PrP-res levels in residual inocula

Given the similar infectivity levels between the brain and spleen homogenate, we analysed the levels of PrP-res present in the residual inocula. Little or no PrP-res was present in the residual brain homogenate from 101LL/GSS and 101LL/263K inocula samples when standard PK digestion and immunoblotting was performed on 10% homogenates (data not shown). NaPTA precipitation was required to detect PrP-res levels in the residual spleen homogenate by increasing the sensitivity levels of detection (19, 20). A low level of PrP-res was present in the 101LL/GSS(a) & (b) residual spleen homogenates (Figure 3B). NaPTA precipitation of the residual brain tissue homogenates (Figure 3A) indicated a similar low level of PrP-res was present in 101LL/GSS(a) & (b) and 101LL/263K(c). However, it is important to highlight that no PrP-res was present following NaPTA precipitation of residual brain or spleen homogenate from 101LL/263K(d) despite this inoculum causing infectious TSE disease in the mice that received the inocula. Indeed no difference was observed in incubation period between 101LL/263K(c) that contained low levels of PrP-res and 101LL/263K(d) that did not contain detectable PrP-res.

Strain properties are maintained on sub-passage with different levels of vacuolation and PrP deposition

The lesion profiles produced from 101LL/GSS and 101LL/263K brain and spleen inoculations (Figure 4) followed a similar pattern varying only in level of vacuolation with higher levels of vacuolation observed from inoculation of the spleen tissue compared to the brain tissue.

Immunohistochemical analysis of the PrP deposition patterns (Figure 5) indicated that PrP deposition targeted similar areas in the recipient brains irrespective of the tissue source of the inoculum. In 101LL/GSS mice, PrP deposition was targeted to the hippocampus, thalamus and the midbrain. In cases with heavier deposition, PrP was also observed in the medulla and occasionally the septum. However, PrP deposition observed was lighter in those mice inoculated with 101LL/GSS spleen homogenate compared to those inoculated with the 101LL/GSS brain homogenate from the same mouse. In 101LL/263K mice, PrP deposition was targeted to the hippocampus with PrP aggregates present in the corpus callosum, midbrain and medulla with similar PrP deposition levels irrespective of the tissue source of the inocula.

Varying levels of PrP-res present in the 101LL/GSS recipient brains dependent on tissue type inoculated.

Given the different levels of PrP deposition observed by immunohistochemistry depending on tissue source for 101LL/GSS, recipient brain tissue from animals receiving brain and spleen inocula was analysed through PK digestion, NaPTA precipitation and immunoblotting. The results (Figure 6) confirm that 101LL/GSS mice that received brain homogenate had a greater level of PrP-res than mice that received spleen homogenate. The differences in the levels of PrP-res in 101LL/GSS were shown to be present in each dilution group inoculated. In contrast, the levels of PrP-res in 101LL/263K tissues were variable independent of tissue-type inoculated whilst the levels of PrP-res in wild-type/79A mice were similar irrespective of the tissue type inoculated. These results were confirmed through a number of different techniques including standard PK digestion (performed at 37˚C without the use of NaPTA) and isolation of scrapie-associated fibrils (data not shown).

DISCUSSION

The prion hypothesis proposes that PrP^{Sc} is the sole or main component of the infectious agent in TSE diseases. Based on the prion hypothesis, the majority of TSE diagnostic tests rely on the detection of PrP-res as indicative of TSE disease. However, recently data have shown that PrP-res and TSE infectivity levels do not always correlate, with infectivity identified in tissues in which PrP-res was not detectable (2, 3, 13, 14). The involvement of the lymphoreticular system in TSE diseases is dependent upon the combination of host species, genotype and TSE agent. In the majority of murine TSE disease models, the levels of PrP-res present in the spleen are lower than those present in the brain tissue (11, 12). However, in the disease models investigated here, we have shown equally short incubation periods following sub-passage of either brain or spleen homogenate from the same mouse, indicating the presence of similar levels of infectivity in both tissues. This was in contrast to the wild-type 79A control which showed consistently extended incubation times with spleen homogenate (30-40 days) compared to brain homogenate. Based on previous data (14), we can therefore hypothesise that despite the extremely low levels of PrP-res identified in both brain and spleen of these mice following NaPTA precipitation the titre of infectivity in each tissue is similar, at approximately10^7-10^9 IU/g. Thus the spleen tissue maintains the discrepancy observed in the brain tissue of these unique disease models with high infectivity levels observed despite the presence of low levels or an absence of PrP-res. Full titration studies are currently planned to accurately establish the infectious titre in the spleen tissues of the 101LL/GSS and 101LL/263K mice. However, the initial data from this disease model prove that TSE agents can have the potential to replicate to high infectivity levels in peripheral tissues when the combination of host species, PrP genotype and TSE agent provides the correct conditions for replication of the infectious agent.

Previous studies of these disease models have shown the presence of little or no PrP-res in brain tissue, despite relatively high levels of infectivity (14, 16, 17). Further investigation here has now demonstrated the presence of low levels of PrP-res in these tissues following concentration of approximately 10mg of tissue homogenate by NaPTA precipitation. Interestingly, the levels of PrP-res detected in brain and spleen of each mouse were similar, and experiments to determine whether this is the truly infectious subpopulation of PrP^{Sc} are on-going in our laboratory. However, despite identifying similar levels of PrP-res in spleens of 101LL/GSS and 79A (Figure 3), estimated levels of infectivity based on incubation times were significantly higher in 101LL/GSS spleen homogenates. Moreover, 101LL/263K(d) was able to transmit disease from both the brain and spleen tissue despite the absence of PrP-res in the residual inocula of both tissues even with the inclusion of NaPTA precipitation as a concentration step to increase the sensitivity levels. Together these data support the continued lack of correlation between PrP-res levels and infectivity in these mouse models of disease.

Given both 101LL/GSS and 101LL/263K had similar incubation periods to disease from both brain and spleen tissue, it can be presumed that this unexpected result was not solely due to the strain of TSE agent or compatibility at codon 101/102. Indeed, the hamster 263K scrapie strain is propagated in animals with proline at PrP codon 102 (equivalent to codon 101 in mice), and does not produce the same disease phenotype when passaged in hamsters. Therefore the disease phenotype in 101LL mice is not an intrinsic characteristic of the strain but rather due to the specific combination of host species, PrP genotype and strain of agent that allows this disease phenotype of high infectivity levels but extremely low levels of PrP-res to manifest. It is possible that this phenotype is uniquely due to the 101L mutation in the recipient mice altering disease pathogenesis or selecting a different isolate from the heterogeneous population of the infectious

307 agent. Further analyses of other TSE strains (e.g. ME7, 79A, 301V) in these mice (which show
308 similar PrP-res levels to wild type mice; (22)) are planned to address this issue.

309 Several recent studies have identified the presence of quasi-species present within individual
310 cases of TSE disease in humans (23, 24), animals (25) and in cell-culture models (26). Indeed it
311 has been hypothesised that sub-variants of disease-associated PrP replicate preferentially in a
312 specific tissue type with dependence on tissue-specific host factors (25, 27). The biochemical
313 and immunohistochemical analysis of the recipient mice from the 101LL/GSS sub-passage
314 demonstrated that PrP-res deposition was lower in the brain tissue of mice that received the
315 spleen homogenate inocula compared to those that received the brain homogenate inocula
316 (Figure 6) whilst in contrast the vacuolation score in mice was greater in those that received the
317 spleen homogenate than the brain homogenate (Figure 4). These results indicate that a potentially
318 heterogeneous population of PrP-res was present. It is hypothesised that tissue-specific
319 conditions supported replication of different sub-variants that upon sub-passage showed different
320 replication efficiencies. If heterogeneous populations of PrP-res exist with variants that have
321 different replication capabilities, a variant present in peripheral tissues may have a higher level
322 of infectivity than the corresponding brain-derived variant. Therefore assessment of the
323 peripheral infectivity levels from novel and emerging isolates is urgently required to ensure an
324 accurate titre is established to maintain food safety.

325 Together these results indicate that a form of the infectious agent may be present in this disease
326 model that remains undetectable by current standard analysis. Given the increasing costs of
327 bioassay to identify the presence of TSE infectivity, the majority of disease cases are being
328 confirmed by biochemical techniques specific for the presence of PrP-res without confirmation
329 of the presence of infectivity. This current reliance on PrP-res as indicative of TSE disease may

not detect all cases of TSE disease with the possible emergence of cases with high infectivity levels associated with low levels or an absence of PrP-res. Indeed the discovery of significant levels of TSE infectivity in the spleens from sheep infected with atypical scrapie despite the absence of PrP-res (13) indicates that this disease phenomenon can occur in natural cases of TSE disease present in the environment. .Further, a recent study by Gonzalez and colleagues highlighted the discrepancy between levels of PrP-res and TSE infectivity in sheep scrapie and sheep BSE and indicated that quantitative laboratory tests to detect disease-associated PrP could not be used to accurately predict infectious titres (28).While TSEs remain in the environment, the emergence of novel isolates, or the possibility that a known isolate could infect a different host species remains. Our data show that a combination of host species, PrP genotype and TSE isolate has the potential to occur that could produce a novel disease phenotype with high levels of TSE infectivity in the absence of PrP-res. Therefore if infectivity levels in the peripheral tissues of disease cases with low levels of PrP-res are higher than originally hypothesised from previous research into classical isolates and current biochemical tests, the emergence of a novel isolate could pose a major risk to food safety if tissues were able to enter the food chain. Together with the discrepancy between PrP-res and TSE infectivity levels presented here, the estimation of titre should not rely on the detection of PrP-res as the sole indicator of TSE disease.

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440 Figure Legends

441 Figure 1: **PrP-res levels in 101LL/GSS and 101LL/263K spleens compared to PrP-res levels**
442 **in wild-type/ME7 spleens**. Spleen homogenates were PK digested, NaPTA precipitated and the
443 samples loaded at a tissue equivalent of 15mg (A and B) or loaded a different tissue weights
444 (mg) as indicated (C and D). PrP-res was present in 101LL/GSS (A) and 101LL/263K (B)
445 spleens with variation between three individual spleens (lanes 1-3). ME7 spleen samples loaded
446 as control (A; Lane 4. B; Lanes 4 and 5). The dilution series (C and D) allowed estimation of the
447 difference in PrP-res levels between 101LL/GSS (C; Lanes 6-9) or 101LL/263K (D; Lanes 6-
448 10) with levels observed in wild-type/ME7 spleens (C and D lanes 1-5). Normal brain
449 homogenate was loaded on each blot to provide PrP^C band as internal control. Blots probed with
450 mAb 8H4 and exposed for 5 minutes.

451

452 Figure 2: **Similar average incubation periods to disease from brain and spleen transmissions**
453 **from 101LL/GSS and 101LL/263K.** In comparison, the average incubation periods for
454 transmission of brain and spleen tissue from wild-type mice were statistically different, with
455 longer incubation periods produced from inoculation with spleen tissue (blue bars). Statistics
456 calculated with student's two-tailed t-test with *p*-values of 2.88×10^{-5}, 3.66×10^{-6}, and 1.08×10^{-8}
457 for 10^{-2}, 10^{-3} and 10^{-4} groups respectively for wild-type/79A transmission. One 101LL/GSS(a)
458 dilution, 10^{-3} produced a significant difference (*p*-value 8.3×10^{-5}) and one 101LL/GSS(b)
459 dilution, 10^{-2} produced a significant difference (*p*-value 5.6×10^{-4}).

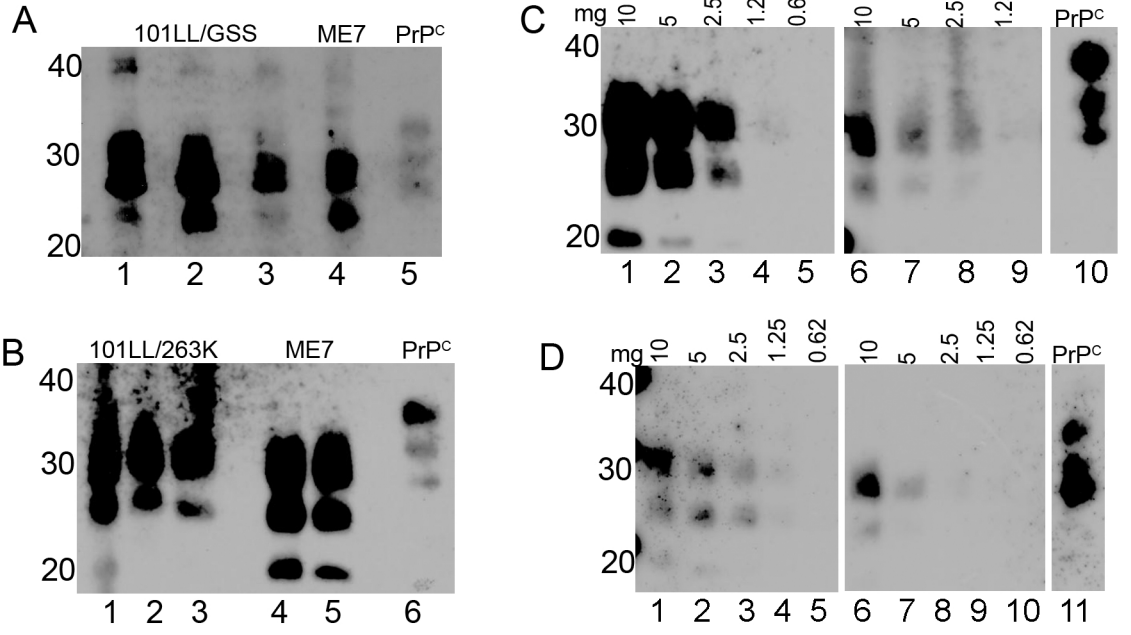
460

Figure 3: **Low PrP-res levels in (A) brain and (B) spleen residual inocula identified by NaPTA precipitation.** In contrast PrP-res was present in wild-type/79A brain and spleen residual homogenates. Residual homogenates were PK digested, NaPTA precipitated. Samples were loaded at a wet weight tissue equivalent of 10mg, proteins separated by SDS-PAGE. Immunoblots probed with mAb 8H4 and exposed for 10 minutes.

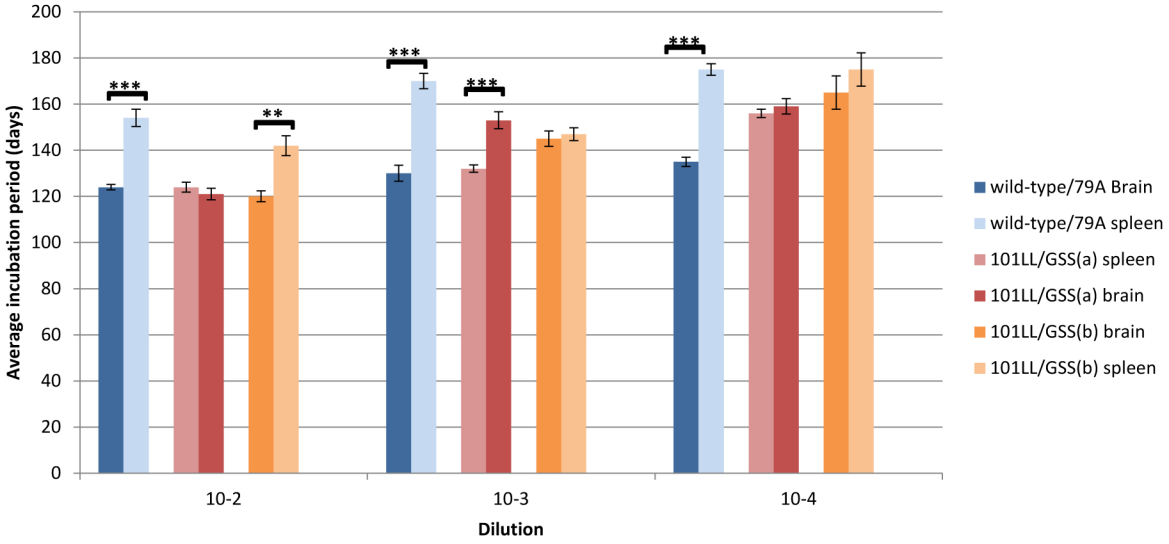
Figure 4: **Similar lesion profiles with slight variation in vacuolation levels dependent on the tissue type inoculated.** Lesion profiles were similar for both brain and spleen tissue inocula in (A) 101LL/GSS(a), (B) 101LL/GSS (b), (C)101LL/263K(c) and (D) 101LL/263K(d) but a higher vacuolation level was observed after inoculation of the spleen tissue compared to the brain tissue. In comparison, the lesion profiles from inoculation of brain and spleen tissue from wild-type/79A mice (E) followed the same pattern with inoculation of the brain tissue producing a slightly higher vacuolation level than the spleen tissue.

Figure 5: **Lighter PrP^{Sc} deposition observed from inoculation of spleen homogenates compared to brain homogenates from 101LL/GSS.** Similar levels of PrP^{Sc} deposition for 101LL/263K irrespective of tissue inoculated. Tissues taken from mice inoculated with 10^{-2} dilution of inocula. Immunohistochemistry performed with mAb 6H4. Hippocampus region are shown at x2 magnification in left-hand column. Brain areas containing PrP^{Sc} deposition are shown at x20 magnification.

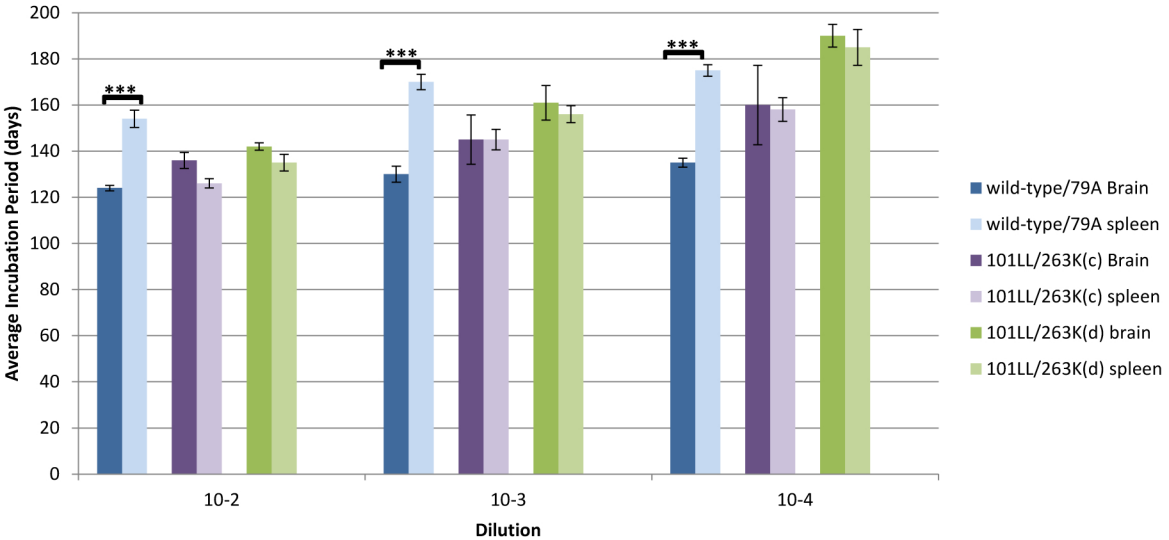
Figure 6: **PrP-res levels in recipient mouse brain vary dependent upon tissue type inoculated from 101LL/GSS mice.** This phenomenon was observed from both (A) 101LL/GSS(a) and 101LL/GSS(b) mice. In contrast, (B) 101LL/263K(c) and 101LL/263K(d) had variable levels of PrP-res independent of tissue-type inoculated whilst (A and B) wild-type/79A recipient brains had the same level of PrP-res independent of tissue-type inoculated. Tissue homogenates were PK digested and NaPTA precipitated. Lanes 1, 2, 5, 6, 9, 10 : Brain tissue. Lanes 3, 4, 7, 8, 11, 12: Spleen tissue. Samples loaded at an equivalent of a wet tissue weight of 33mg. An uninfected brain homogenate was loaded at 10mg/ml (w/v) wet weight tissue in lane 13 as internal control. Blots probed with mAb 8H4.

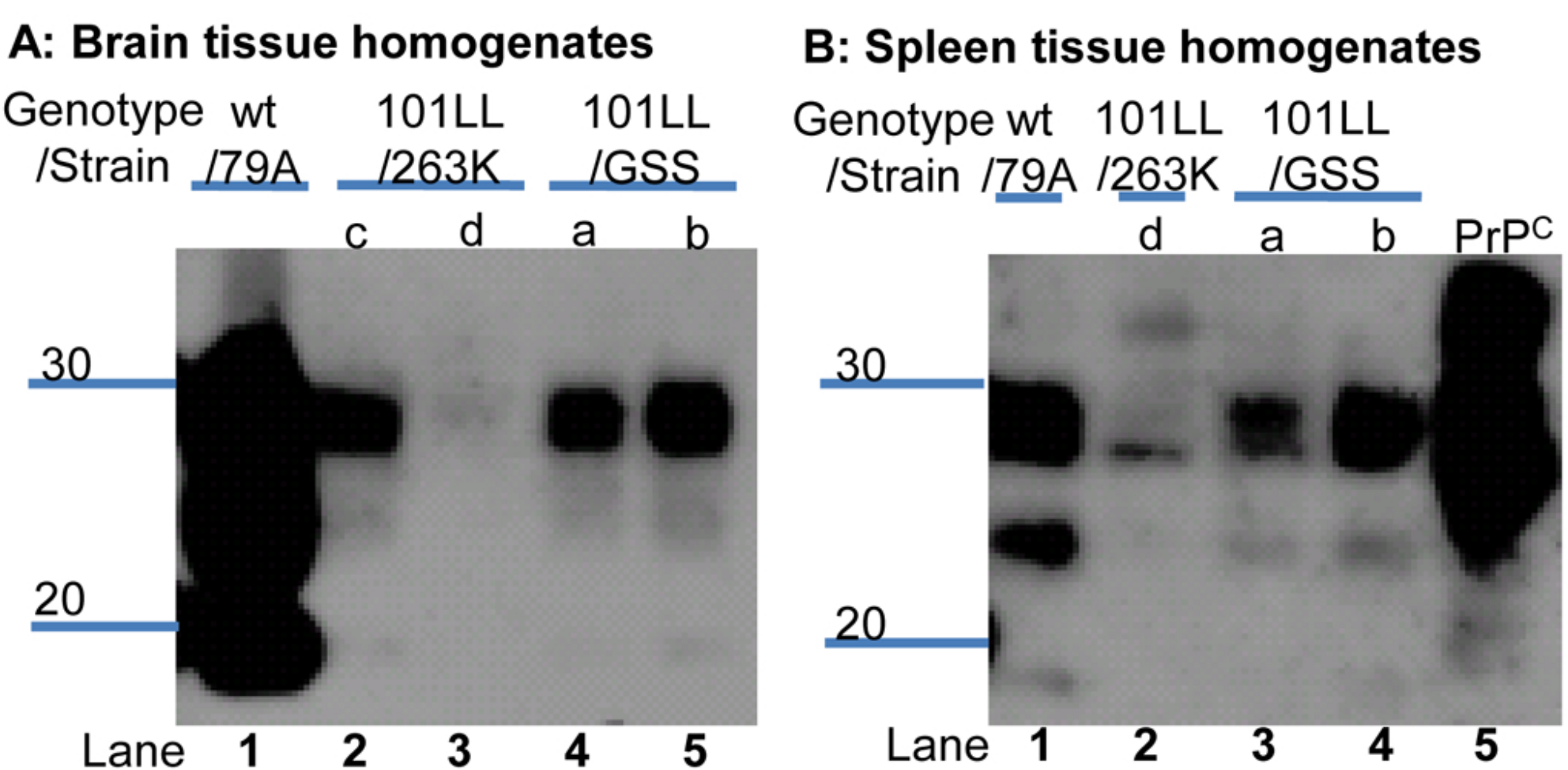

A
101LL/GSS
ME7
PrPC
40
30
20
1 2 3 4 5
B
101LL/263K
ME7
PrPC
40
30
20
1 2 3 4 5 6
C
mg 10 5 2.5 1.25 0.62
10 5 2.5 1.25
PrPC
40
30
20
1 2 3 4 5 6 7 8 9 10
D
mg 10 5 2.5 1.25 0.62
10 5 2.5 1.25 0.62
PrPC
40
30
20
1 2 3 4 5 6 7 8 9 10 11

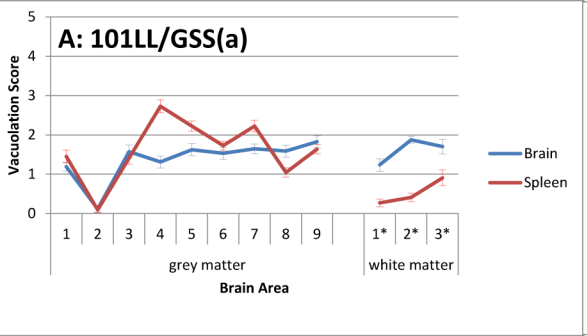
A: 101LL/GSS brain and spleen incubation periods

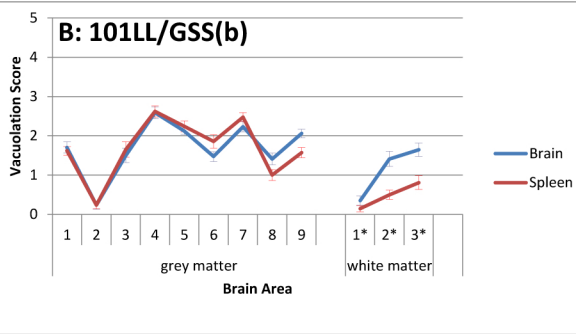


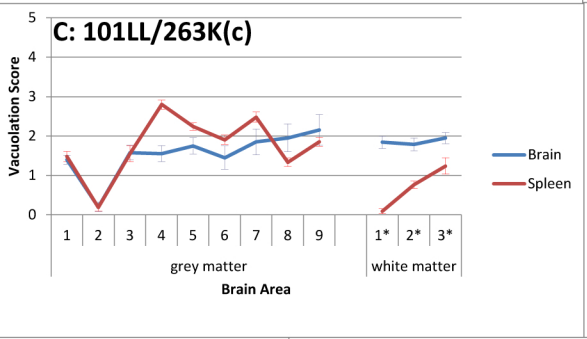
B: 101LL/263K brain and spleen incubation periods

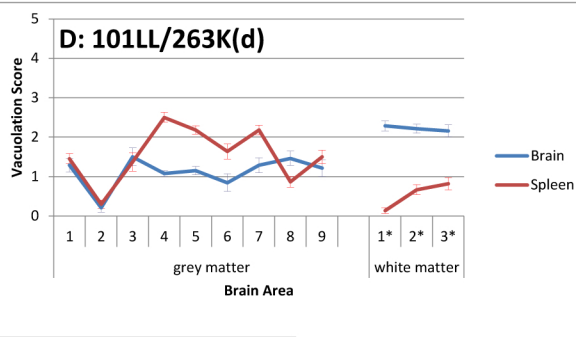


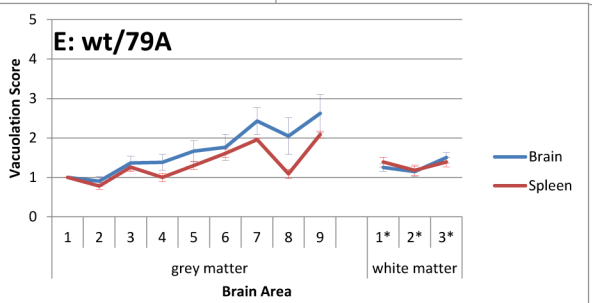

A: Brain tissue homogenates
Genotype /Strain
wt /79A
101LL /263K
101LL /GSS
c
d
a
b
30
20
Lane 1 2 3 4 5
B: Spleen tissue homogenates
Genotype /Strain
wt /79A
101LL /263K
101LL /GSS
d
a
b
PrPC
30
20
Lane 1 2 3 4 5

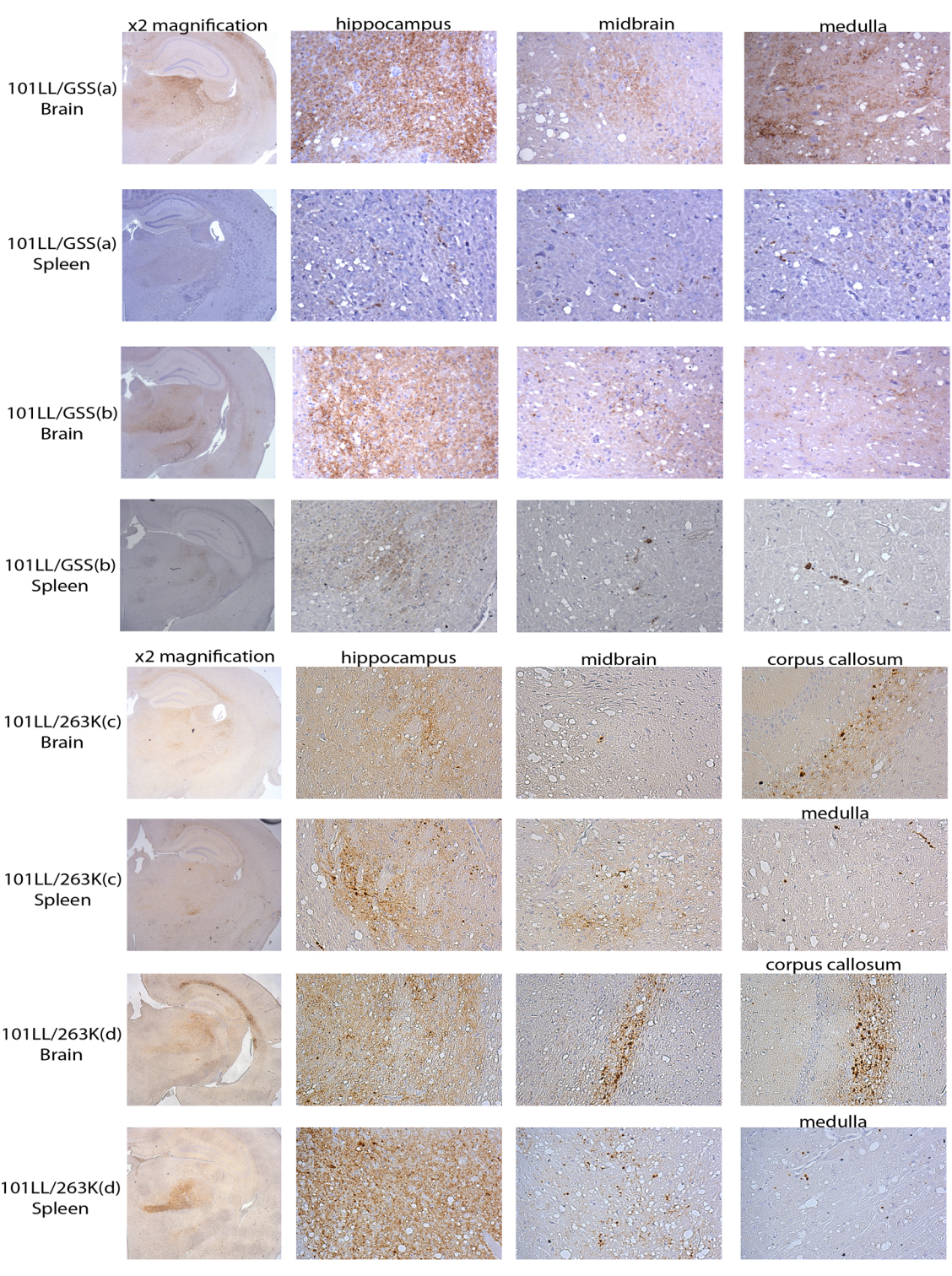

A: 101LL/GSS(a)
Vacuolation Score
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
1*
2*
3*
grey matter
white matter
Brain Area
Brain
Spleen

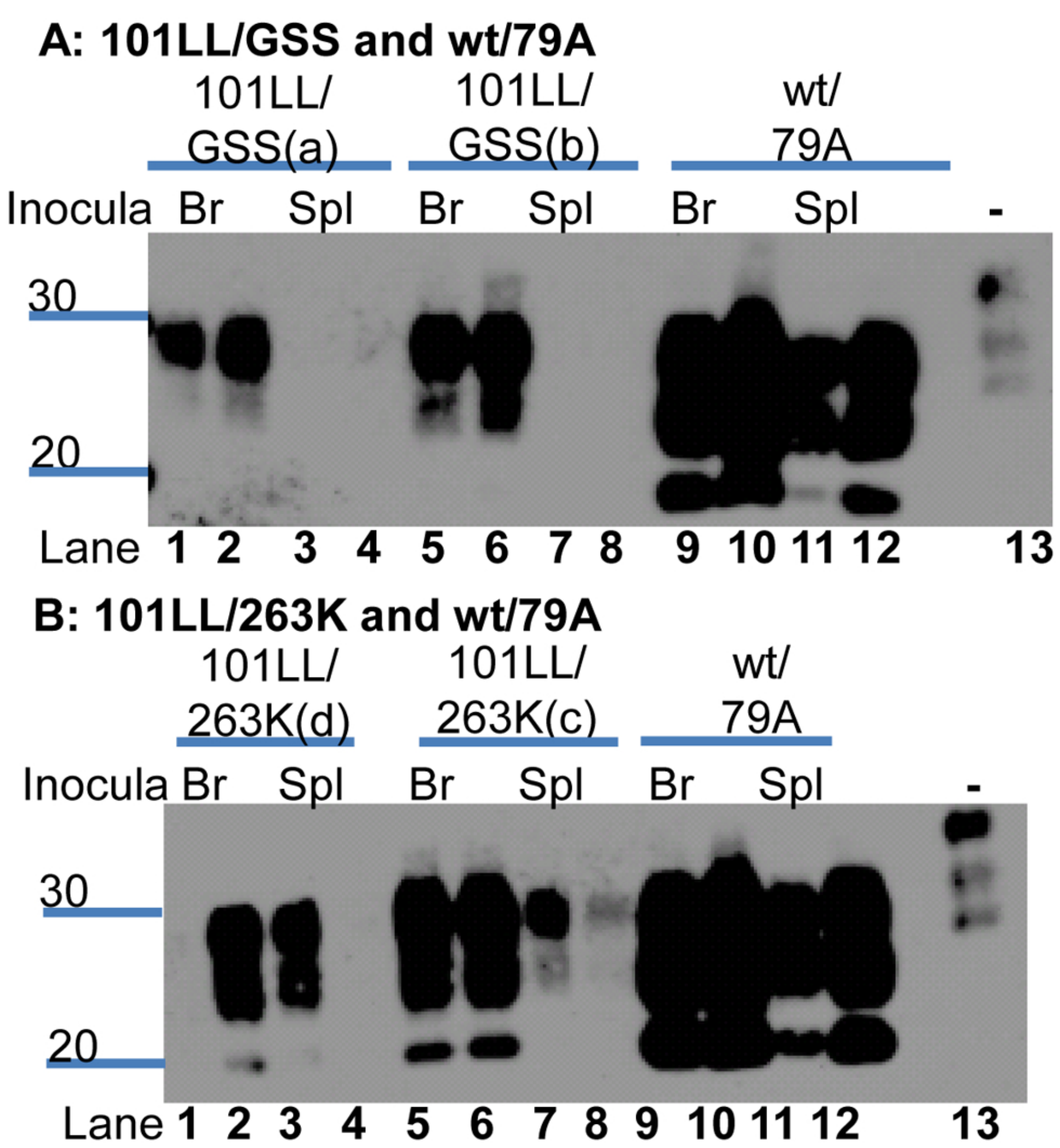

B: 101LL/GSS(b)
Vacuolation Score
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
1*
2*
3*
grey matter
white matter
Brain Area
Brain
Spleen


C: 101LL/263K(c)
Vacuolation Score
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
1*
2*
3*
grey matter
white matter
Brain Area
Brain
Spleen


D: 101LL/263K(d)
Vacuolation Score
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
1*
2*
3*
grey matter
white matter
Brain Area
Brain
Spleen


E: wt/79A
Vacuolation Score
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
1*
2*
3*
grey matter
white matter
Brain Area
Brain
Spleen


x2 magnification
hippocampus
midbrain
medulla
101LL/GSS(a)
Brain
101LL/GSS(a)
Spleen
101LL/GSS(b)
Brain
101LL/GSS(b)
Spleen
x2 magnification
hippocampus
midbrain
corpus callosum
101LL/263K(c)
Brain
medulla
101LL/263K(c)
Spleen
corpus callosum
101LL/263K(d)
Brain
medulla
101LL/263K(d)
Spleen


A: 101LL/GSS and wt/79A
101LL/ GSS(a)
101LL/ GSS(b)
wt/ 79A
Inocula Br Spl Br Spl Br Spl -
30
20
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
B: 101LL/263K and wt/79A
101LL/ 263K(d)
101LL/ 263K(c)
wt/ 79A
Inocula Br Spl Br Spl Br Spl -
30
20
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13